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Cryptococcus allantoinivorans sp.nov., an anamorphic basidiomycetous yeast (Tremellales) physiologically resembling other species of the *Cryptococcus laurentii* complex that degrade polysaccharides and C2 compounds

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Abstract

A novel *Cryptococcus* species is proposed to accommodate a yeast strain (CBS 9604) able to assimilate allantoin as sole carbon source, a characteristic very uncommon among yeasts. By traditional methods, the strain could not be distinguished from *Cryptococcus laurentii*, but nucleotide sequences of the D1D2 region of the large subunit (26S) and of the ITS region of ribosomal DNA showed relationship to the *Bulleromyces* clade of the genus *Cryptococcus* (order Tremellales) with some *Tremella* spp. as the closest relatives. A traditional morphological and physiological description of the strain is given. Data on the assimilation of some C2 compounds and polysaccharides are provided and compared with those of other type strains of novel species of the *C. laurentii* complex.

Introduction

In nature a great variety of organic compounds that contain only two carbon atoms (C2 compounds) are present. Many of these are readily assimilated by several yeast species or are building blocks of biomass. Examples are ethanol, acetate and glycine. Assimilation of ethylamine as sole nitrogen source is a well-known taxonomic characteristic (van der Walt 1962), shown by the majority of yeast species studied. Assimilation of ethylamine as sole carbon source has been reported also (Middelhoven et al. 1985, 1986) but ethanolamine supported growth of only one yeast strain, CBS 7140, identified as *Cryptococcus laurentii* (Middelhoven et al. 1985, 1986). Ethanolamine is wide-spread in nature as a building block of phospholipids. Enrichment cultures on ethanolamine inoculated with soil yielded only one yeast strain able to utilize the hydroxyamine as sole carbon source, but on aerial plant surfaces such yeasts are more common.

Uric acid as sole carbon source supported growth of several ascomycetous and basidiomycetous yeasts (Middelhoven et al. 1983, 1984, 1985). In *Candida famata* and *Trichosporon cutaneum* uric acid is oxidized to allantoin which in three successive hydrolytic steps is converted into two moles of urea and one mole of glyoxylate from which biomass is synthesized and energy is generated (Middelhoven et al. 1983). Hence, uric acid, considered from a physiological viewpoint, is a C2 compound. Yeast strains that assimilated uric acid as sole carbon source failed to grow on the oxidation product allantoin, some strains of *Stephanoascus ciferrii* excepted (Middelhoven et

al. 1985). At present, it can not be explained why uric acid positive yeasts did not grow on allantoin, as the latter is a good nitrogen source for many strains. Many attempts to isolate yeasts from soil by enrichment culture on allantoin as sole carbon source failed (unpublished results). Eventually, strain CBS 9604 was isolated from a rotten mushroom. Mushrooms contain allantoin (Brunel 1931, 1936).

Based on unique biochemical features and on nucleotide base sequences differing from those of *C. laurentii*, a novel species, *Cryptococcus allantoinivorans*, is proposed to accommodate strain CBS 9604 that is the type strain.

Materials and Methods

Strain *C. laurentii* CBS 7140 had been isolated from enrichment cultures with ethanolamine as sole carbon source (Middelhoven et al. 1985) inoculated with soil. Strain CBS 9604 was isolated from an enrichment culture with allantoin (2 g per litre) as sole carbon source, inoculated with a rotten mushroom *Hericium erinaceus* that grew on the trunk of a beech tree in Wageningen, The Netherlands, September 2000 (Middelhoven 2004a). Strains were maintained on Potato Dextrose Agar (Difco) slant cultures.

The strains were examined for morphological and physiological properties with standard yeast identification methods (Yarrow 1998). Utilization of carbon and nitrogen sources was tested in liquid Yeast Nitrogen Base and Yeast Carbon Base (Difco), respectively, but nitrite assimilation was tested by the auxanographic technique. The pH of growth media was adjusted to pH 5.5 as required, but the pH of media with galacturonic and quinic acids was not adjusted, which is in agreement with the laboratory practice of the CBS (D.Yarrow, personal communication; Middelhoven 1997). Growth on D-glucarate (saccharate), L-malate, galactarate (mucic acid) and tartaric acids was tested at pH 4.0.

In addition to standard substrates, several non-traditional compounds were tested as growth substrate (5 g per litre) for the type strains listed in Table 1. These tests were carried out in a basal growth medium (GB) (Middelhoven et al. 1991) that had the composition of Yeast Nitrogen Base but the phosphate concentration was tenfold higher to improve the buffering capacity and the nitrogen source, when present, was 2 g ammonium chloride per litre, rather than 5 g of ammonium sulphate that is used in Yeast Nitrogen Base. A droplet of a pre-culture in 2 ml of glucose (5 g per litre) basal growth medium was inoculated into 2 ml medium in culture tubes of 150 mm, 19 mm width that were incubated in a rotary shaker at 25 °C. No ammonium chloride was added to growth media with nitrogenous substrates, such as uric acid, glycine, allantoin, ethanolamine, ethylamine, L-phenylalanine and L-4-hydroxyproline. Cultures on uric acid (10 g per litre, pH not adjusted) were agitated vigorously to keep the crystals in suspension. Sparse, but discernible growth at the expense of this energy-poor and weakly soluble substrate was observed after disappearance of the crystals. Growth on potentially toxic phenolic compounds and on n-hexadecane was observed in slant cultures (Middelhoven et al. 1991, 2004). Growth on polygalacturonate and tannic acid was tested in GB medium at pH 5.5; starch, inulin, dextran (0.25%), pullulan (0.25%), galactomannan (0.25%), and birch xylan (1.0 %, insoluble fraction removed by centrifugation) in GB medium at pH 6.5.

All molecular sequencing and DNA analyses were performed at the University of Miami by Dr. J.W. Fell and Dr. G. Scorzetti (Fell et al. 2000; Scorzetti et al. 2002).

Table 1. Strains studied

C. allantoinivorans Middelhoven, CBS 9604^T, from decaying mushroom
C. aureus Takashima et al., CBS 318^T, from atmosphere
C. carnescens Takashima et al., CBS 973^T, from muscatel grape
C. flavescens (Saito)Skinner, CBS 942, from atmosphere
C. laurentii (Kufferath)C.E.Skinner, JCM 9066^T (= CBS 139^T), from palm wine; CBS 2174, from a tumour; CBS 8648, from lung; CBS 7140, from soil (enrichment on ethanolamine)
C. nemorosus VKM Y-2906^T (= CBS 9606^T), from turf, Moscow region, Russia
C. peneaus Takashima et al., CBS 2409^T, from surface of shrimp
C. perniciosus VKM Y-2905^T (= CBS 9605^T), from herbaceous plants in oak forest, Moscow region, Russia
Papiliotrema bandonii Sampaio et al., VKM Y-2917^T (= CBS 9107^T), fruit bodies on a pyrenomycete parasiting on grass in Portugal

Results and Discussion

Strains of *C. laurentii* present in the CBS culture collection differ in many characteristics traditionally used in yeast taxonomy (Kurtzman and Fell 1998; Barnett et al. 2000). Takashima et al. (2003) reclassified some of these strains based on sequence analysis

of 18S rDNA, the D1D2 region of the 26S rDNA and the ITS region and defined three novel species, viz. *Cryptococcus aureus, C. carnescens* and *C. peneaus*. In an earlier study a novel teleomorphic yeast, physiologically similar to *C. laurentii,* had been described, viz. *Papiliotrema bandonii* (Sampaio et al. 2002). Golubev et al. (2003) described two novel *Cryptococcus* species, closely related to *Papiliotrema*, viz. *Cryptococcus nemorosus* and *Cryptococcus perniciosus*. *C. allantoinivorans* is related to the latter group of species, known as the *Bulleromyces* clade. These species are phylogenetically closely related to *C. laurentii*, except *C. peneaus* and *C. carnescens* that are in the *Cryptococcus victoriae* clade (Golubev et al. 2003; Takashima et al. 2003). Type strains of nine species of the *C. laurentii* complex were subjected to growth tests on some non-traditional carbon cources, viz. C2 compounds like allantoin, ethanolamine etc., some phenolic compounds, L-4-hydroxyproline, L-phenylalanine and some polysaccharides in an attempt to demonstrate the usefulness of these characteristics in the distinction of these taxa. In earlier studies (Middelhoven 2003, 2004b; Middelhoven et al. 2004) some of these growth tests separated *Trichosporon* species.

Strains CBS 7140 and CBS 9604 could not be distinguished from *C. laurentii*, as described by Kurtzman and Fell (1998) and by Barnett et al. (2000), by conventional yeast identification methods. Sequencing of the D1D2 region of the 26S subunit of ribosomal DNA and of the ITS region revealed that CBS 7140 differed from *C. laurentii* CBS 139^T by only one base pair in D1D2 and ten in ITS. Nucleotide sequences of the D1D2 and ITS domains of the large subunit (26S) ribosomal DNA of CBS 7140 were deposited at GenBank, accession numbers AY 315663 for D1D2 and AY 315665 for ITS. Both strains were considered to be conspecific. A detailed study of physiological characteristics of strains of *C. laurentii* sensu Takashima et al. (2003) (Table 2, Table 3) showed large physiological similarity, but the strains varied in assimilation of glycerol, glucono-δ-lactone, 5-ketogluconate, L-tartrate, D-tartrate, *meso*-tartrate, Tween 60, Tween 80, creatine (N-source), polygalacturonate and tannic acid, in tolerance to 10% NaCl and in growth at 37 °C. Ethanolamine was assimilated by all strains of *C. laurentii* sensu Takashima et al. (2003). This character was also shown by *C. nemorosus, C. perniciosus* and *C. flavescens*. Except for *C. laurentii* CBS 7140 and *C. nemorosus* these strains were no inhabitants of soil.

CBS 9604 was subsequently shown out to represent an unknown species. In the D1D2 region *Papiliotrema bandonii* (Tremellales) was the closest match, but a phylogenetic analysis showed relationship to some *Tremella* spp, belonging to the *Bulleromyces* clade of the genus *Cryptococcus* (Figure 1). For this reason a novel species, *C. allantoinivorans*, is proposed to accommodate this strain. For lack of more strains, mating experiments were not carried out.

Latin diagnosis of Cryptococcus allantoinivorans Middelhoven

In medio liquido dextrosum et peptonum et extractum levidinis et extractum malti continente post 3 dies 25 °C cellulae globosae ad ovoideae (6.2-8.5 × 6.5-10 μm),singulae vel binae, hyphae et pellicula non formatur. Sedimentum album formatur, quod etiam post 4 hebdomades adest. Cultura in agaro PDA dicto post 3 dies albida, butyrosa, nitida, non elevata; post hebdomades 4, 20 °C, eadem forma. In agaro extracto malti confecto post dies 3, 20 °C, hyphae non formantur. Fermentatio nulla. D-Glucosum, D-galactosum, L-sorbosum (lente), D-glucosaminum, acetyl-D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, trehalosum, α-methyl-D-glucosidum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum (lente), raffinosum, melezitosum, inulinum (lente), glycerolum, erythritolum, ribitolum, xylitolum, L-arabinitolum, D-glucitolum, mannitolum, galactitolum, inositolum, gluconolactonum, acidum gluconicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum glucuronicum, acidum lacticum, acidum succinicum, acidum citricum, aethanolum, propano-1,2-diolum, acidum glucaricum et acidum galactonicum assimilantur. Amylum solubile, acidum galacturonicum, methanolum, butano-2,3-diolum, acidum quinicum non assimilantur. Aethylaminum, L-lysinum, cadaverinum, creatinum (lente), creatininum, D-glucosaminum (lente) et D-tryptophanum (lente) assimilantur, neque kalii nitratum, natrii nitritum, imidazolum. Thiaminum externum crescentiae necessarium. Reactio Diazonii coerulei B positiva. 32 °C crescit neque 35 °C. Ureum finditur. Materia amyloidea formatur. Typus CBS 9604, isolatus ex *Hericium erinaceus* in Wageningen, Neerlandia, lyophilus praeservatus in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

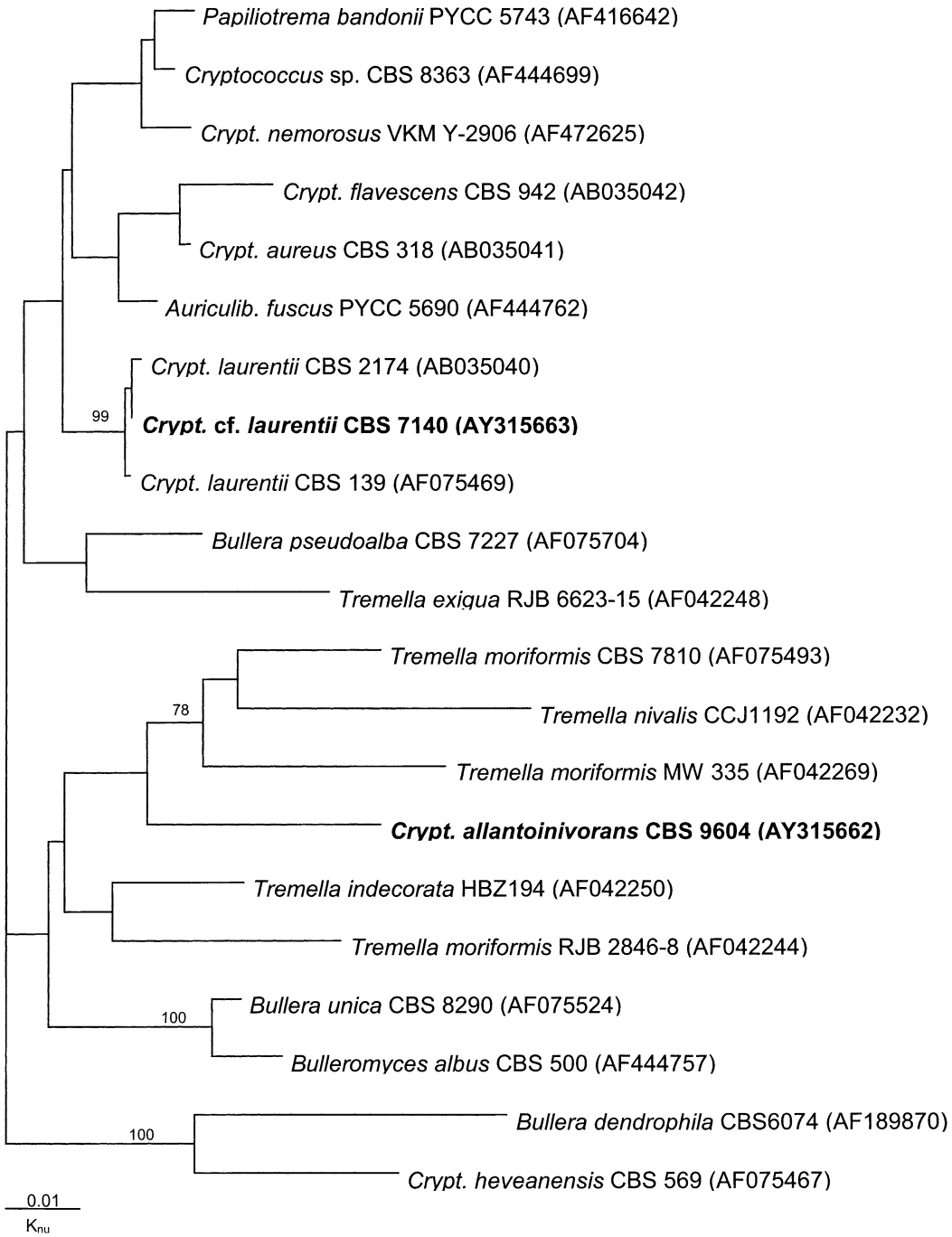

Figure 1. Phylogenetic tree of *Cryptococcus allantoininvorans* and of selected hymenomycetous yeasts in the Tremellales lineage obtained by Neighbour-Joining analysis of aligned 26S rRNA gene (D1/D2 domains) sequences using PAUP 4.0b8 (evolutionary distance calculated according to Kimura). The numbers given on the branches are the frequencies ($>75\%$) with which a given branch appeared in 1000 bootstrap replications. *B. dendrophila* and *C. heveanensis* were used as outgroup. Sequences determined in the present study are typed in boldface. Additional sequences were retrieved from GenBank (accession numbers between parentheses).

Table 2. Physiological characteristics of *Cryptococcus allantoinivorans* CBS 9604 and *C. laurentii* sensu Takashima et al. (2003)

Strains studied	*C. allantoinivorans*	*C. laurentii* sensu Takashima et al. (2003)			
	CBS 9604^{T}	139$^{T,1)}$	2174$^{1)}$	8648$^{1)}$	7140
Carbon compounds					
D-Glucose	+	+	+	+	+
D-Galactose	+	+	+	+	+
L-Sorbose	D	–	DW-	–	–
D-Glucosamine	+	+	+	+	+
D-Ribose	+	D	+	+	+
D-Xylose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Arabinose	+	+	+	+	+
L-Rhamnose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
αα-Trehalose	+	WD++	+	+	+
Methyl-α-glucoside	+	+	+	+	+
Cellobiose	+	+	+	+	+
Salicin	+	+	D	+	+
Arbutin	+	+	+	+	+
Melibiose	+	+	+	+	+
Lactose	D	+	+	+	+
Raffinose	+	+	+	+	+
Melezitose	+	+	+	+	+
Inulin	D	–	DW-	–	W
Soluble starch	–	+	+	+	+
Glycerol	+	–	–	+	+
meso-Erythritol	+	+	+	+	+
Ribitol	+	+	+	+	+
Xylitol	+	+	+	+	+
L-Arabinitol	+	+	+	+	+
D-Glucitol	+	+	DW	+	DW
D-Mannitol	+	+	+	+	+
Galactitol	+	+	+	+	+
myo-Inositol	+	+	+	+	+
Glucono-δ-lactone	+	DW	+	+	+
2-Ketogluconate	+	+	+	+	+
5-Ketogluconate	+	–	–	?	+
D-Gluconate	+	+	+	+	+
D-Glucuronate	+	+	+	+	+
Galacturonic acid	+	DW	+	+	+
DL-Lactate	+	D	+	D	+
Succinate	+	+	+	+	+
Citrate	+	+	+	+	+
Methanol	–	–	–	–	–
Ethanol	+	DW	+	+	+
Propane-1,2-diol	+	–	–	–	–
Butane-2,3-diol	–	–	–	–	–
Quinic acid	–	–	–	–	–
D-Glucarate (saccharate)	+	D	+	+	+
D-Galactonate	+	+	+	+	+
Palatinose	+	+	+	?	+
Levulinate	–	–	–	?	–
L-Malate	+	+	+	?	+
L-Tartrate	D	DW	D	?	+
D-Tartrate	DW	–	+	?	+
meso-Tartrate	–	–	DW	?	D

Table 2. Continued.

Strains studied	*C. allantoinivorans*	*C. laurentii* sensu Takashima et al. (2003)			
	CBS 9604^{T}	139$^{T,1)}$	2174$^{1)}$	8648$^{1)}$	7140
Galactaric (Mucic) acid	+	+	+	?	+
Gentobiose	+	+	+	?	+
Ethyleneglycol	–	–	–	?	–
Tween 60	D	–	+	?	+
Tween 80	D	–	–	?	+
N-acetyl-D-glucosamine	+	+	+	?	+
Fermentation					
Glucose	–	–	–	–	–
Nitrogen compounds					
Nitrate	–	–	–	–	–
Nitrite	–	–	–	–	–
Ethylamine	+	+	+	+	+
L-Lysine	+	+	+	+	+
Cadaverine	+	+	+	+	+
Creatine	D	+	+	–	+
Creatinine	+	+	+	+	+
D-Glucosamine	D	+	+	W	D
Imidazole	–	–	–	–	–
D-Tryptophan	D	–	–	–	+
D-Proline	D	+	+	?	+
Putrescine	+	+	+	?	+
Urotropine	+	+	D	?	+
Miscellaneous					
Vitamins	Th	Th	Th	?	Th
0.01% Cycloheximide	+	DW	+	D	+
O.1% Cycloheximide	D	–	–	D	–
10% NaCl, 5% Glucose	+	+	+	–	–
16% NaCl, 5% Glucose	–	–	–	–	–
Growth at pH 3.0	+	+	+	?	+
Growth at pH 9.5	–	–	–	?	–
Amyloid production	+	+	+	+	+
Urease	+	+	+	+	+
DBB stain	+	+	+	+	+
Max.T °C	32+35	− 35+37-	35+37-	37+40-	37+40-

+, growth within 8 days; −, no growth after 20 days; D, growth after 8 days or more; W, weak growth response; Th, thiamine required; ?, unknown, not done; [1)]data from CBS Yeast Data Base and own observations.

Characteristics of C. allantoinivorans

After 3 days growth in liquid growth medium containing glucose (1%, wt/vol), yeast extract (0.3%, wt/vol), peptone (0.5%, wt/vol), malt extract (0.3%, wt/vol) at 25 °C cells are globose to ovoid (6.2-8.5 × 6.5-10 μm). A sediment is present but a pellicle is not formed. The slant culture on Potato Dextrose Agar (PDA agar) after 3 days is of butyrous texture, tends to flow down and is white and glistening. The appearance does not change over 4 weeks. Slide cultures on malt extract agar, PDA agar and Yeast Morphology Agar did not show hyphae but globose to ovoid cells only. Physiological characteristics of strain CBS 9604 were listed in Table 2.

Origin and deposits

Strain CBS 9604 was isolated by W.J. Middelhoven from an enrichment culture on allantoin as sole carbon source, inoculated with a rotten mushroom *Hericium erinaceus* (Bull.:Fr.)Pers. that grew on the trunk of a beech tree, *Fagus sylvatica* L. in Wageningen, The Netherlands, September 2000. Nucleotide sequences of the D1D2 and ITS domains of the large subunit (26S) ribosomal DNA of CBS 9604 were de-

Table 3. Diagnostic growth tests of 11 strains of the *Cryptococcus laurentii* complex.

Growth substrate	1	2	3	4	5	6	7	8	9	10	11
Ethanol	+	D	+	+	D	+	+	+	–	–	–
Ethylamine	–	–	–	–	–	–	–	+	–	–	–
Ethanolamine	–	+	+	+	–	+	+	–	+	–	–
Glycine	D	+	+	+	+	+	+	+	–	–	–
Allantoin	+	–	–	–	–	–	–	–	–	–	–
L-4-Hydroxyproline	+	–	–	–	+	–	–	+	–	–	–
Starch	–	D	+	+	D	+	+	D	–	–	–
Pullulan	–	D	D	+	D	+	+	–	+	+	+
Dextran	–	–	–	–	–	–	–	–	–	–	+
Xylan	+	+	–	+	+	+	+	+	–	+	+
Inulin	D	–	–	–	–	–	–	–	–	–	–
Polygalacturonate	+	+	–	+	+	+	–	–	–	–	D
Tannic acid	–	–	–	+	–	–	–	–	–	–	–
Phloroglucinol	–	–	–	–	–	+	–	–	–	–	–

+, growth within 8 days at 20 °C;, D, growth after 8–20 days; −, no growth within 20 days; Compounds tested but not supporting growth of any strain: glycollate, glyoxylate, ethyleneglycol, ethylenediamine, butylamine, uric acid, galactomannan, hexadecane, several phenolic compounds; 1, *Cryptococcus allantoinivorans* CBS 9604^T; 2, *Cryptococcus laurentii* JCM 9066^T (= CBS 139^T); 3, *C. laurentii* CBS 2174; 4, *C. laurentii* CBS 7140; 5, *Papiliotrema bandonii* VKM Y-2917^T (CBS 9107^T); 6, *C. nemorosus* VKM Y-2906^T (CBS 9606^T); 7, *C. perniciosus* VKM Y-2905^T (CBS 9605^T); 8, *C. flavescens* CBS 942^T; 9, *C. aureus* CBS 318^T; 10, *C. peneaus* CBS 2409^T; 11, *C. carnescens* CBS 973^T.

posited at GenBank, accession numbers AY 315662 for D1D2 and AY 315664 for ITS.

Etymology. The epithet allantoini-vorans refers to the ability to assimilate allantoin as sole carbon source

Physiological similarity of *C. allantoinivorans* CBS 9604 and strains of *C. laurentii* sensu Takashima et al. (2003) is shown in Table 2. Of all conventional characters tested, both species differed only in assimilation of L-sorbose, soluble starch, inulin, galacturonic acid, propane-1,2-diol and D-tryptophan, in tolerance to 10% NaCl and in growth at 35 °C, in total less than 10% of the tests done.

In Table 3 growth tests of 9 species of the *C. laurentii* complex on non-conventional substrates are recorded. *C. laurentii* and *C. allantoinivorans* differed in assimilation of ethanolamine, allantoin, L-4-hydroxyproline and pullulan, that is in 4 of the 12 tests done. The strains of *C. laurentii* sensu Takashima et al. (2003) behaved similarly, showing variable growth responses to tannic acid and polygalacturonate only. Tannic acid was assimilated only by the soil-inhabiting strain CBS 7140. The other seven species investigated showed characteristic growth responses to C2 compounds, polysaccharides and some other carbon sources and could be distinguished from each other and from *C. laurentii* and *C. allantoinivorans* by the growth tests recorded in Table 3. These turned out to be valuable tools in the delimitation of these species and possibly of other *Cryptococcus* species. Some tests, e.g., assimilation of uric acid, galactomannan, L-phenylalanine and phenolic compounds, were not useful as growth responses of all strains tested were negative. This is in contrast to recent studies of the genus *Trichosporon* (Middelhoven 2003, 2004b; Middelhoven et al. 2004) in which some of these tests separated clades and species.

Many aromatic compounds were not assimilated by the strains studied. These included phenol, hydroquinone, resorcinol, 4-methylcatechol, orcinol, 3-hydroxybenzoate, gentisate, protocatechuate, 4-hydroxyphenylacetate, 4-hydroxycinnamate and 4-hydroxyacetophenone. However, *C. nemorosus* assimilated phloroglucinol. This confirms the conclusions of another report (Sampaio 1999) that Tremellales do not or poorly grow at the expense of benzene compounds.

The present study shows that isolation of yeast strains by enrichment on non-traditional carbon sources, followed by nuclear base sequencing, is an efficient way to detect novel species in nature, that otherwise would be overlooked. Growth tests on polysaccharides and other non-conventional carbon compounds separate basidiomycetous yeasts but the species treated in this study require another group of compounds than saprophytic *Trichosporon* species (Middelhoven 2004).

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